



Development of a prototype wound dressing technology which can detect and report colonization by pathogenic bacteria

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ABSTRACT

A new methodology for detecting the microbiological state of a wound dressing in terms of its colonization with pathogenic bacteria such as *Staphylococcus aureus* or *Pseudomonas aeruginosa* has been developed. Here we report how stabilized lipid vesicles containing self-quenched carboxyfluorescein dye are sensitive to lysis only by toxins/virulence factors from *P. aeruginosa* and *S. aureus* but not by a non-toxic *Escherichia coli* species. The development of the stabilized vesicles is discussed and their response to detergent (triton), bacterial toxin (α -hemolysin) and lipases (phospholipase A₂). Finally, fabrics with stabilized vesicles attached via plasma deposited maleic anhydride coupling are shown visibly responding to *S. aureus* (MSSA 476) and *P. aeruginosa* (PAO1) but not *E. coli* DH5 α in a prototype dressing.

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1. Introduction

The evolved resistance exhibited by numerous bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA), in addition to the inherent antibiotic resistance of bacteria such as *Pseudomonas aeruginosa* in biofilms, has led to the reconsideration of how infections are controlled and treated, particularly in clinical environments [Meyer et al., 2010]. At the time of writing, whilst effective antibiotics for a range of infections still exist, their use is now more carefully controlled to prevent evolved resistance. With the evolution of ever more antibiotic-resistant bacteria, there is an urgent requirement for the effective diagnosis of bacterial infection, thus preventing antibiotic overuse [WHO, 2001].

Burns and other wounds are particularly susceptible to bacterial infection as they bypass the body's primary defence mechanism, the skin, allowing direct bacterial ingress into underlying tissue. The diagnosis of infection is often self-evident: inflammation around the wound site, obvious wound colonization, pyrexia and inflammatory laboratory tests. There are, however, clinical conditions where diagnosis is not straightforward and where speed of detection is essential, for example in burns of young children under the age of five years [White et al., 2005]. A disproportionate number of scald injuries relative to the overall population occur in young

children: in the United Kingdom, 53% of all scald burns occur in this age range, yet they make up only 6% of the population. It is this group of children that are also particularly likely to suffer burn-related infections due to a lack of immunity. Antibody protection to certain bacterial toxins increases with age, with children under five years having only 30% compared to adult levels [Childs et al., 1999]. Young children are therefore more likely to suffer from complications arising from primary infection by *Staphylococcus* or *Streptococcus* species. For example, Toxic Shock Syndrome (TSS), caused by the 'super'-antigen present in certain bacteria such as some *S. aureus* and *Streptococcus* species can result in an over-activation of the patient's immune system. If not treated TSS can lead to death within hours [Young and Thornton, 2007]. Wound swabs, microbiological cultures and/or other related tests used to diagnose infection often do not provide clinicians with the timely guidance they require, resulting in inaccurate diagnoses based on the child's clinical condition alone.

There is therefore a critical clinical requirement to diagnose infection at early stages preferably without the removal of adherent dressings. A wound dressing that is designed to change colour, could indicate the change in microbiological state, allowing for rapid intervention. Methods for doing this have been proposed in the literature, with pH being postulated as an indicator of wound health. Changes in pH have been observed as a wound heals, although there is high inter-patient variation and differences observed between acute wounds, burns and chronic wounds [Gethin, 2007]. Another approach being considered as a "wound environ-

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ment indicator” is that of electronic ‘noses’ that measure the gas composition above wounds, thus detecting infection in the wound [Byun et al., 2010].

Using vesicles as drug delivery vehicles has had much attention over recent years, with interest extending into utilizing vesicles in both cancer chemotherapy and antimicrobial delivery [An and Friedman, 1998; Falkow et al., 1992; Van Amersfoort et al., 2003]. Griset et al. described the synthesis and testing of pH sensitive polymeric nanoparticles which respond to their local environment [Griset et al., 2009]. At pH 7.4, the hydrogel nanoparticles were stable, at pH 5, the polymer hydrolysed and the particle swelled to up to six times its original diameter and in doing so released an encapsulated drug, paclitaxel, used as a chemotherapeutic agent against lung carcinomas. Mi et al. have investigated a chitosan bilayer in a wound dressing that was reported to give a burst response, followed by slow release of the topical antimicrobial silver sulfadiazine [Mi et al., 2002]. This system has the potential advantage of combining the reported wound healing properties of chitin/chitosan with antibiotic action. However, there are reports that the presence of silver ions in wounds can actively reduce wound healing, with silver being cytotoxic at concentrations required for it to be an effective antimicrobial [Poon and Burd, 2004]. Vesicles have been shown to be able to transfer drugs through skin (transdermally) and to the lungs [Bouwstra and Honeywell-Nguyen, 2002; Lu et al., 2003]. Attempts at using vesicles to deliver drugs within the blood stream have had to combat a more difficult environment including various immune responses – both innate and acquired – designed to target foreign bodies such as the complement cascade and phagocytosis [Funato et al., 1994].

The results discussed in this paper follow on from a concept paper published in 2010, which showed that lipid vesicle bilayer membranes could behave as substrates to bacterial toxins and virulence factors in a similar way to eukaryotic cell membranes, being permeabilized or hydrolysed by toxins and enzymes secreted by key pathogenic bacteria in wounds such as α -hemolysin or phospholipase A_2 from *S. aureus* and *P. aeruginosa* [Zhou et al., 2010]. In this paper methods to stabilize phospholipid vesicles, whilst retaining their sensitivity to bacterial toxins, using photo-polymerizable fatty acid moieties are discussed and a working, prototype detection system for pathogenic bacteria on a simple model wound dressing is shown.

2. Materials and methods

2.1. Vesicle production

Vesicles contained 20 mol% cholesterol (Sigma–Aldrich), 2 mol% 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine (DMPE) and varying fractions of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) (Avanti) and 10,12-tricosadiynoic acid (TCDA) (Sigma–Aldrich). Lipids, cholesterol and TCDA were mixed in chloroform, dried under nitrogen before being hydrated in 5 ml Tris buffer pH 7.5 containing 10 mmol dm^{-3} 5(6)-carboxyfluorescein (from Sigma–Aldrich) via vigorous mixing and then being left overnight at 5°C to allow lipid head groups to hydrate and for membrane components to separate into phase domains. The vesicles were then purified on a Sephadex G-25 column (GE Healthcare) to separate non-encapsulated dye from vesicles before being exposed to UV light (254 nm) for a total of 60 s from a high intensity UV source (Hamamatsu photonics).

2.2. Microbiology

Pathogenic bacteria used in the experiment were clinically isolated strains of methicillin-susceptible *S. aureus* (MSSA 476) and *P.*

aeruginosa (PAO1), with a lab strain (non-pathogenic bacterium) of *Escherichia coli* (DH5 α) with most virulence factors removed and used as a control. Bacterial cultured LB media was prepared in MilliQ water and autoclaved before usage. Bacteria were grown in 10 ml LB for 16 h on a shaker incubator at 37°C . The optical density (OD) of bacterial cultured LB medium was measured at 600 nm following growth. OD measurements for the bacteria after 16 h growth (in stationary phase) were: *S. aureus* = 1.970; *P. aeruginosa* = 1.466; *E. coli* = 1.378. Bacteria were diluted in LB prior to experiments, with inoculation concentrations of 1000 CFU ml^{-1} being used.

2.3. Pathogenicity assay

The basis of the detection assay is that at high concentration, carboxyfluorescein is non-fluorescent. Following breakdown of the lipid vesicle containers by bacterial toxins, the dye becomes diluted and “switches on”. Experiments were performed on a 96 well plate using a BMG Labtech fluorostar plate reading fluorimeter. A $10 \mu\text{l}$ vesicle solution was added to $190 \mu\text{l}$ of bacteria (10^6 CFU ml^{-1}) in LB culture media on a 96 well plate, allowing all experiments to be repeated 6 times for a given set of conditions. The dye was excited with light at 495 nm, and emission measured at 520 nm. Plates were incubated at 37°C and shaken every 30 s for 2 s prior to measurement. Fluorescence was recorded every 4 min and plotted both vs. time and as a histogram at a fixed time point, showing both the absolute value of fluorescence and increase following addition of Triton X-100 to lyse the vesicles.

Bacterial growth in 96 well plates was measured with a BMG Labtechfluorostar in absorbance mode, using a software correction factor to plot absorbance data at 600 nm for 1 cm path length. As with fluorescence measurements, the plates were incubated at 37°C and shaken before measurement. Due to the broad spectrum of absorbance of carboxyfluorescein which swamped the bacterial scattering absorbance measurement at 600 nm, bacterial growth and lysis measurements were performed on two separate instruments, but side by side, with the same bacterial culture and experimental conditions except presence or absence of fluorescein.

2.4. Attachment of vesicles to fabric

The attachment of giant unilamellar vesicles containing DMPE lipids, using plasma deposited maleic anhydride, has been described in a previous publication [Liu et al., 2005]. A 13.56 MHz RF source was used to create a thin film of pulse plasma polymerized maleic anhydride on polypropylene fabric with retention of the anhydride group functionality. Immediately following film deposition, the fabric was immersed in an aqueous suspension of the lipid vesicles for 30 min allowing formation of amide linkages between the anhydride group and amine functionality on PE lipids. After rinsing in buffer, the modified fabrics were photographed under a low power (8 W) long wavelength UV lamp with a Nikon digital SLR camera. Three swatches of fabric were studied: (1) vesicles made directly prior to utilization, and attached directly, following maleic anhydride deposition; (2) vesicles allowed to partially dry in the 96 well plate for 48 h after fabrication, before re-hydration in buffer and attachment; (3) attached vesicles from (2) exposed to Triton X-100 detergent. Fig. 1 schematically illustrates the assumed mechanism of action.

The next stage of the work involved testing the response of the optimized (in terms of sensitivity and stability) vesicle system in suspension to strains of the test bacteria. 10^5 CFU ml^{-1} start inoculum of a diluted overnight culture of the *S. aureus*, *P. aeruginosa* and *E. coli* in LB broth ($180 \mu\text{l}$) were added to $20 \mu\text{l}$ of vesicles in HEPES buffer) and release of encapsulated dye measured as the bacteria grew using a fluorometric plate reader recorded. Finally, a crude ‘proof of principle’ prototype was devised, using the plasma

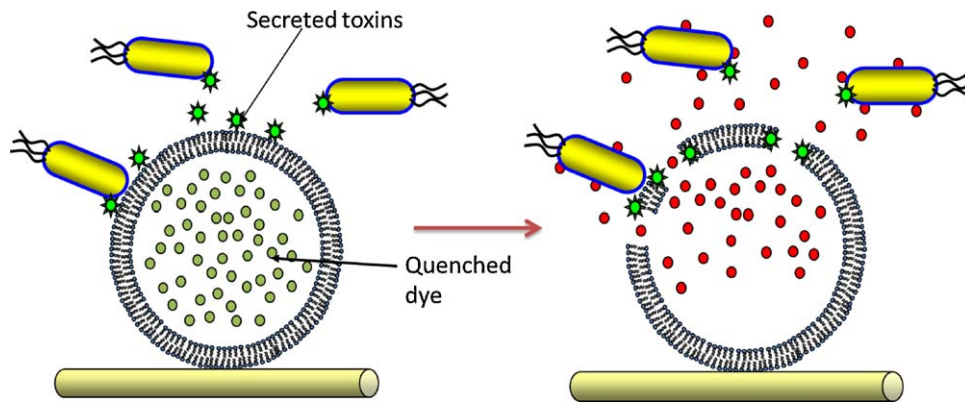


Fig. 1. Schematic showing attached vesicle containing self-quenched carboxyfluorescein dye being lysed by secretion toxins from bacteria resulting in dye release and fluorescence 'switch on'.

polymerized maleic anhydride coupling methodology described above. Swatches of modified fabric were inoculated with overnight culture of the test bacteria, the control being inoculated with HEPES buffer and the fluorescence release photographed under low power UV light after 15 min.

3. Results and discussion

3.1. Vesicle stability and toxin sensitivity

The first part of this study looked at producing relatively stable lipid vesicles which would survive attachment to fabric, part drying and re-hydration, but still respond to pathogenic bacteria by breaking apart and releasing their payload, a fluorescent dye (carboxyfluorescein). Within the vesicle and prior to release the dye is self-quenched. Following release, and subsequent dilution in the wound environment, the dye 'switches on', becoming fluorescent. The stability of the vesicles is a key concern in lipid-vesicle-based sensor systems, both within an aqueous environment and following drying and re-hydration. A number of vesicle stabilization approaches have been discussed in the literature, including the use of sugars and photo-polymerizable lipids [Yavlovich et al., 2011; Maurer et al., 2001]. In this paper, we follow on from the work

of Koushevra et al. using acetylenictricosadiynoic acid (TCDA) [Kolusheva et al., 2000].

Initial experiments studied the effect of the mol% of the photo-polymerizable fatty acid TCDA on the stability of vesicles following partial drying for 48 h, and re-hydration in buffer (Fig. 2).

The data demonstrates that higher concentrations of the TCDA stabilize the vesicle structure, with progressively smaller increases in fluorescence on re-hydration. This suggests that the vesicles retain their structure over the drying/rehydration cycle. The data does not give a clear indication of the vesicle sensitivity to lysing molecules, the detergents or toxins vary with TCDA concentration. It is possible that an over-abundance of TCDA in the membrane may lower the sensitivity of the vesicle to lytic agents, and/or alter the vesicle size and shape such that they cannot carry a sufficient fluorescent payload. This possibility was tested by measuring the sensitivity of the different vesicles to lysis by Triton and two lytic bacterial toxins: α -hemolysin and phospholipase A₂ (PLA₂), Fig. 3. The plot indicates that as the TCDA concentration increases from 0 to 40%, the fluorescent increase upon lysis by Triton, hemolysin or PLA₂ decreases from a four-fold increase at 20% TCDA to a less than two-fold increase at 40%.

3.2. Response of vesicles during growth of *S. aureus*

In order to illustrate the correlation between bacteria concentration (measured as optical density) and fluorescence response of the

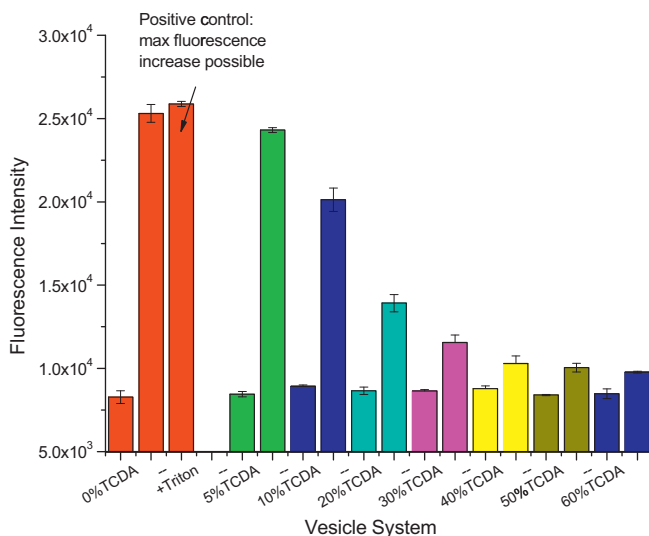


Fig. 2. Vesicle stability: changes in the initial fluorescence of vesicles (left bar of pair) and fluorescence increase following partial drying and rehydration in buffer (right bar) as a function of TCDA mol% in vesicles. The 0% TCDA result indicates the maximum expected fluorescence if all vesicles are lysed by detergent, Triton.

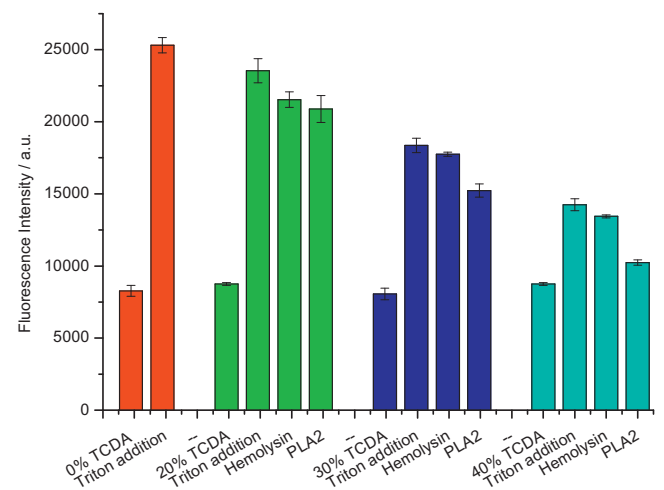


Fig. 3. Vesicle sensitivity to Triton, α -hemolysin and phospholipase A₂ (PLA₂) as a function of TCDA mol%. The 0% TCDA gives the maximum fluorescence change possible for full lysis in absence of TCDA. For each system, the greater change in fluorescence on adding lytic toxin, the higher the sensitivity.

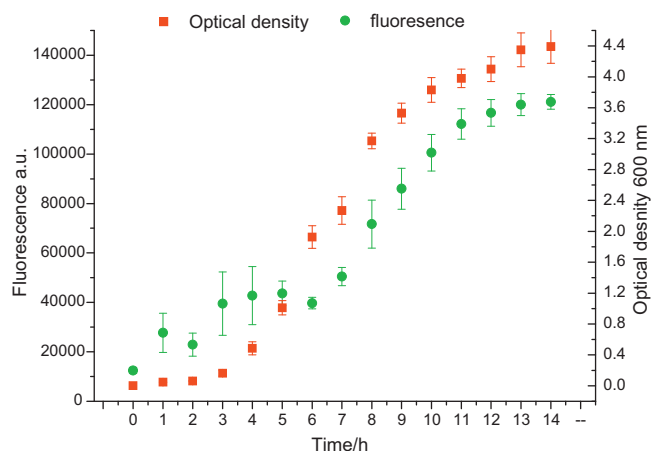


Fig. 4. Plot of optical density of *S. aureus* MSA 476 and concomitant fluorescence change due to vesicle lysis after inoculation with 1000 CFU ml⁻¹ bacteria in LB broth.

vesicles (in suspension), the bacterial optical density at 600 nm and vesicle fluorescence were followed over an 14 h growth of *S. aureus* MSA 476, following inoculation of 10³ CFU ml⁻¹ bacteria into LB. This was carried out using the experimental conditions described previously, and with vesicles containing 20 mol% TCDA. The results are shown in Fig. 4, with optical density plotted on the right vertical axis and fluorescence on the left. It can be seen that 1 h after inoculation, the measured fluorescence has more than doubled: from 12,000 units to 27,000 units, this change also being visible by eye. This suggests that an approximate limit of visible detection for this *S. aureus* strain is between 10⁴ and 10⁵ CFU ml⁻¹.

3.3. Attachment of vesicles to fabric and stability/response testing

This negative correlation with respect to TCDA concentration was visually observed on vesicles attached via pulse plasma deposited maleic anhydride thin films on non-woven polypropylene fabric (Fig. 5). Fig. 3 shows a visual response that correlates with

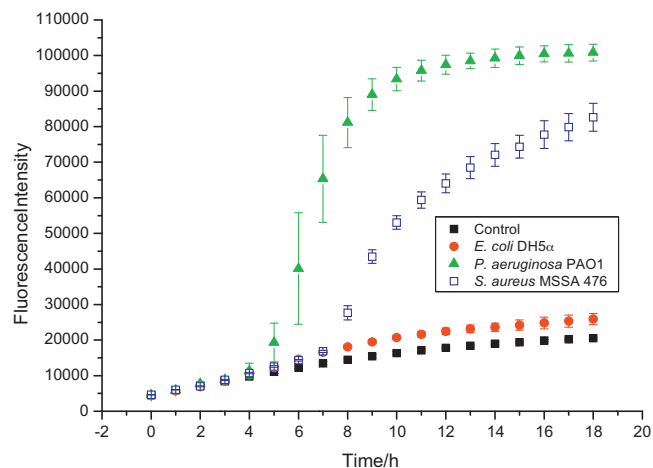


Fig. 6. Time-resolved fluorescence release from 30% TCDA vesicle inoculated with 1000 CFU ml⁻¹ *P. aeruginosa* PAO1, *S. aureus*, MSA 476, *E. coli* DH5α and a non-inoculated control over 18 h.

the measurements in Figs. 2 and 3: too low a TCDA concentration results in the vesicles not being stable to partial drying (0, 5, 10%); too high a concentration of TCDA results in vesicles not being sensitive to lysis by Triton (50% and 60%). The optimum TCDA concentration for stable and sensitive vesicles therefore appears to be between 20% and 30%.

The sensitivity of the vesicles to bacteria and, importantly, their ability to discriminate between the non-pathogenic *E. coli* strain from the two pathogenic strains (*P. aeruginosa* PAO1 and *S. aureus* MSA 476) was tested next. Fig. 6 illustrates the fluorescence release from vesicles as bacteria grow and release toxins. Note that these experiments were performed on vesicles suspended in buffer/LB broth – and not yet attached to fabric.

Fig. 6 demonstrates that the vesicles only responded to the two pathogenic species of bacteria as they grow (*S. aureus* and *P. aeruginosa*), but not to the non-pathogenic *E. coli*. Separate measurements (by optical density) of bacterial growth showed that all

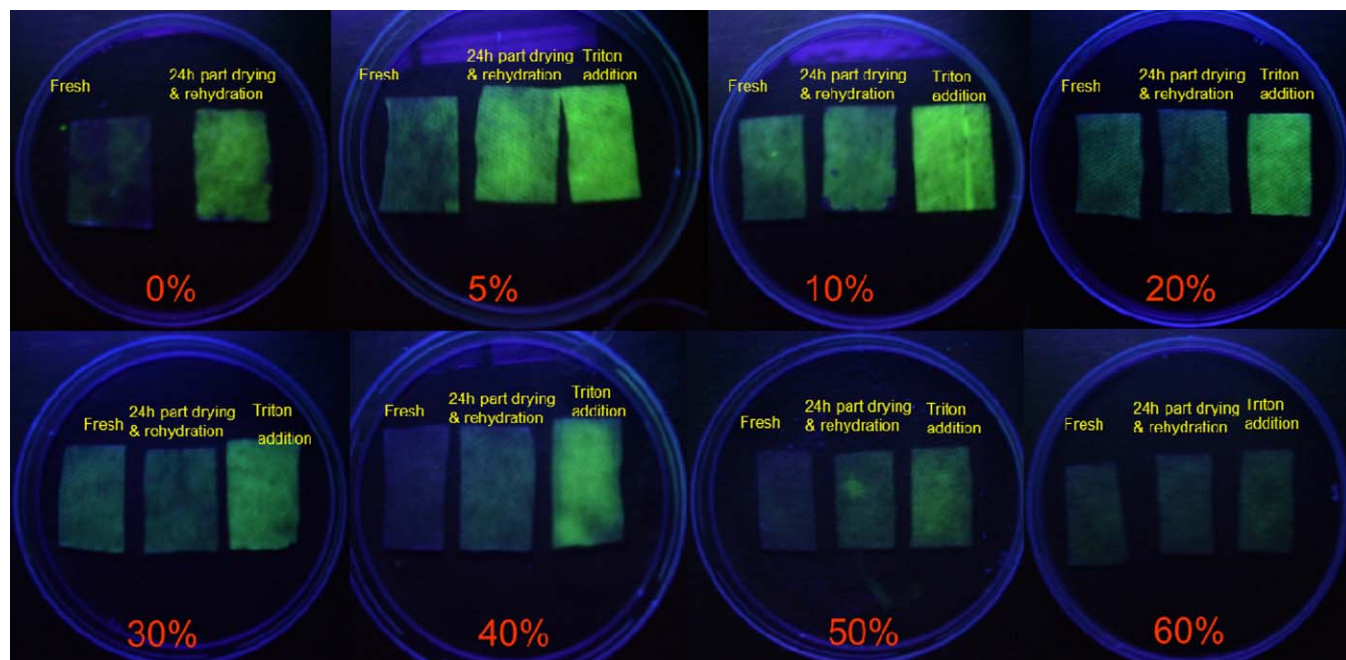


Fig. 5. Polypropylene modified with vesicles containing initially self-quenched carboxyfluorescein (left-hand swatch) and switch on of fluorescence of dye following either vesicle destruction following drying or direct lysis by Triton, as a function of mol% TCDA.

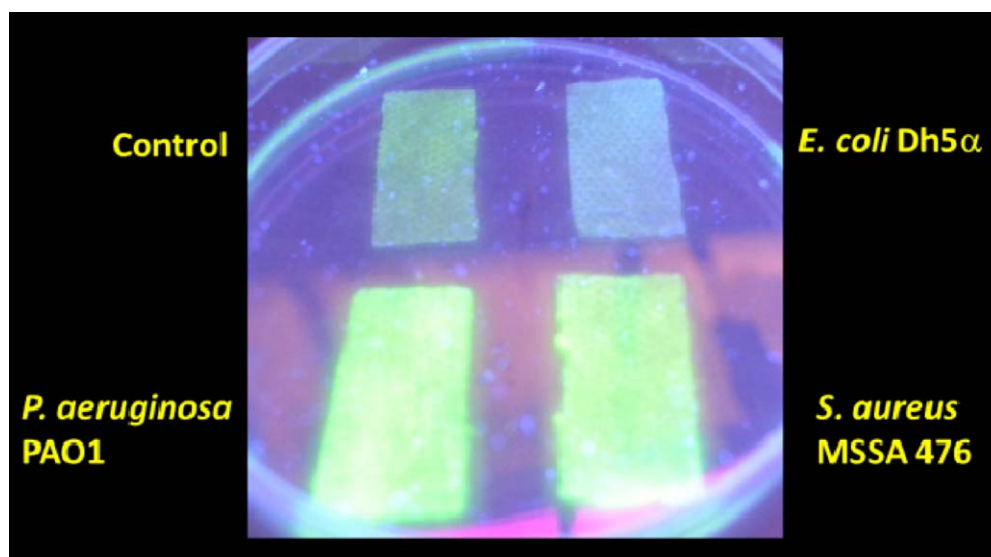


Fig. 7. Simple prototype dressings: response of modified fabric following overnight growth of bacteria on polypropylene fabric with 30 mol% TCDA vesicles.

species grew in the LB broth, but the *P. aeruginosa* is either more virulent, or secretes more toxins during its exponential growth phase than *S. aureus*. The results shown in Figs. 2, 3, 5 and 6 demonstrate that a relatively stable vesicle system can be fabricated using an optimum concentration of TCDA as a stabilizer. The results further illustrate that the vesicles can be coated on to fabric and are sensitive to pathogenic bacteria when dispersed in buffer/broth, “switching on” as the microbes grow. The final part of the study examined whether vesicle-modified fabric would be sufficiently sensitive to pathogenic bacteria to give a visual response under weak UV light, whilst *not* responding to non-pathogenic *E. coli* (Fig. 7). It should be noted that the assay has been tested against a large number of *S. aureus* strains (over 100 clinical isolates), as well as a number of *P. aeruginosa* strains. A good correlation between bacterial toxicity to cultured T-cells and the degree of fluorescence ‘switch on’ was observed (T cells representing a generic mammalian eukaryotic host in this case). This work is on-going and will be published in a more specialised publication in 2012, but preliminary data is provided in the supporting information. This data suggests that for the assay is sensitive to the 96% of a random strain collection of clinical isolates of *S. aureus* (96/100).

This experiment, using pulse-plasma-deposited maleic anhydride to bind the vesicles to the fabric, and to create a thin hydrogel film, gave a clear indication that a fabric could be produced that exhibited a fluorescent response to pathogenic bacteria. In this communication, a concept has been described for a simple pathogenic bacteria sensor in a crude prototype wound dressing.

Principal target pathogens in wounds secrete lytic toxins, which damage healthy tissue. The basis of the sensor-discrimination utilizes this lytic action to cleave walls of the lipid vesicles, which contains a self-quenched dye. Whilst this mode of action is not seen in all pathogenic bacteria: *Vibrio cholerae* for example secretes cholera toxin which is endocytosed by target gut endothelial cells interfering with cell machinery [Holmgren, 1981], it is a mode of action broadly exhibited by common pathogens present in burn wounds: *P. aeruginosa* and *S. aureus* [Branski et al., 2009]. Arguably, one of the actions which make skin and wound bacteria acutely pathogenic is their ability to damage host tissue. Thus infection by non-lytic bacteria – which would not activate this putative sensor system – would be of less clinical concern than an acutely lytic bacterial species or strain. Vesicles with 30 mol% TCDA are relatively more resistant to damage arising from partial drying than in the

absence of TCDA, but will not survive in a completely dry environment. Recent results show that vesicles remain intact and stable in hydrogels such as gelatin for greater than 30 days. Various methods for creating hydrogels, which assist wound healing and stabilize the vesicles, are currently being studied and will be reported in a subsequent paper.

4. Conclusions

This paper describes a methodology for wound state sensing which might have a broad applicability beyond burns. The current focus is on developing manufacturable prototype dressings to be taken forward for clinical trials within the next four years, combining the antimicrobial release technology described in an earlier article with the fluorescent ‘switch on’ reporting of infection described here to produce an advanced dressing which both treats infection at source but also monitors infection in the wound [Zhou et al., 2010; Jenkins and Young, 2010].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.08.028.

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